Table 1. The probability of forming a hydrogen bond between the 97th aspartic acid in L4/5 and the 119th arginine in L6/7 of the CA in 60,000 trajectories during 5–20 nanoseconds of MD simulations and the sensitivity phenotype.

120th amino acid	Frequency of hydrogen bond (%)	Sensitivity to CM TRIM5α
Pro (P)	44.6	Sensitive
Phe (F)	41.5	Sensitive
His (H)	42.99	Sensitive
Ile (I)	0	Sensitive
Ala (A)	64.47	Resistant
Gln (Q)	55.15	Resistant
Asn (N)	55.7	Resistant
Glu (E)	21.27	Resistant
Ser (S)	63.51	Resistant
Thr (T)	51.48	Resistant

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position of the TRIM5α-resistant viruses GH123/Q (D97A-GH123/Q) and GH123/A (D97A-GH123/A). The side chain of A at the 97th position would be too small to form a hydrogen bond with the 119th R, which was confirmed by MD simulation study of the D97A CA mutant of GH123/Q (Figure 6). As expected, the D97A substitution conferred moderate sensitivity to CM TRIM5α upon the resistant viruses GH123/Q and GH123/A (Figure 7A and 7B). In the case of TRIM5α-sensitive virus GH123/P, in which the probability of hydrogen bond formation between the 97th D and 119th R was predicted to be low (Table 1), the D97A substitution did not alter the viral senstivity to CM TRIM5α (Figure 7C). These data suggest that the conformation of L4/5, which is influenced by that of L6/7, participates in determining viral sensitivities to CM TRIM5α-mediated restriction. It should be noted, however, that the D97A substitution slightly impaired the replication of GH123/Q and GH123/A, as indicated by the titers of D97A-GH123/Q and D97A-GH123/A, which were apparently lower than those of GH123/Q and GH123/A at day 5 after infection even in the absence of TRIM5α (Figure 7A and 7B). Although we further tried to disrupt the hydrogen bond formation by introducing an alanine substitution at the 119th position, the resultant mutant viruses did not grow (data not shown). The arginine at the 119th position is highly conserved among different HIV-2 strains and may be essential for virus replication.

In the case of the TRIM5α-resistant virus GH123/E (Figure 4, gray model), however, the conformation of L4/5 was similar to those of CM TRIM5α-sensitive viruses GH123/P, GH123/F, GH123/H and GH123/I (Figure 4, red models). The probability of hydrogen bond formation was also low in GH123/E, unlike that in the other resistant viruses GH123/Q, GH123/A and GH123/N (Table 1). Because GH123/E has a negatively charged amino acid E at the 120th position, we performed additional modeling of the CM TRIM5α-resistant virus with another negatively charged amino acid D (GH123/D). The results showed that the conformation of GH123/D L4/5 was also similar to those of CM TRIM5α-sensitive viruses (data not shown). Consistent with this, the possibility of hydrogen bond formation was low (21.27%) in GH123/D just as in GH123/E. It

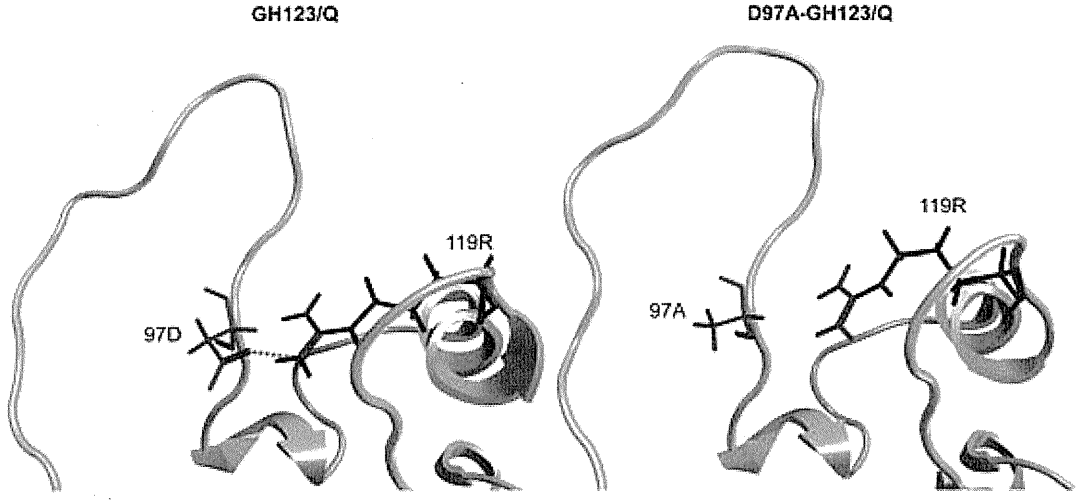

Figure 6. Lack of hydrogen bond formation between the 97th alanine and the 119th arginine of HIV-2 D97A-GH123/Q CA. Close-up views of averaged structures around the L4/5 loop of GH123/Q (left) and D97A-GH123/Q (right) during 5–20 nanoseconds of MD simulations are shown. Red, blue and green wireframes denote side chains of aspartic acid at the 97th (97D), arginine at the 119th (119R), and alanine at the 97th (97A) positions, respectively. A dotted line indicates a hydrogen bond.
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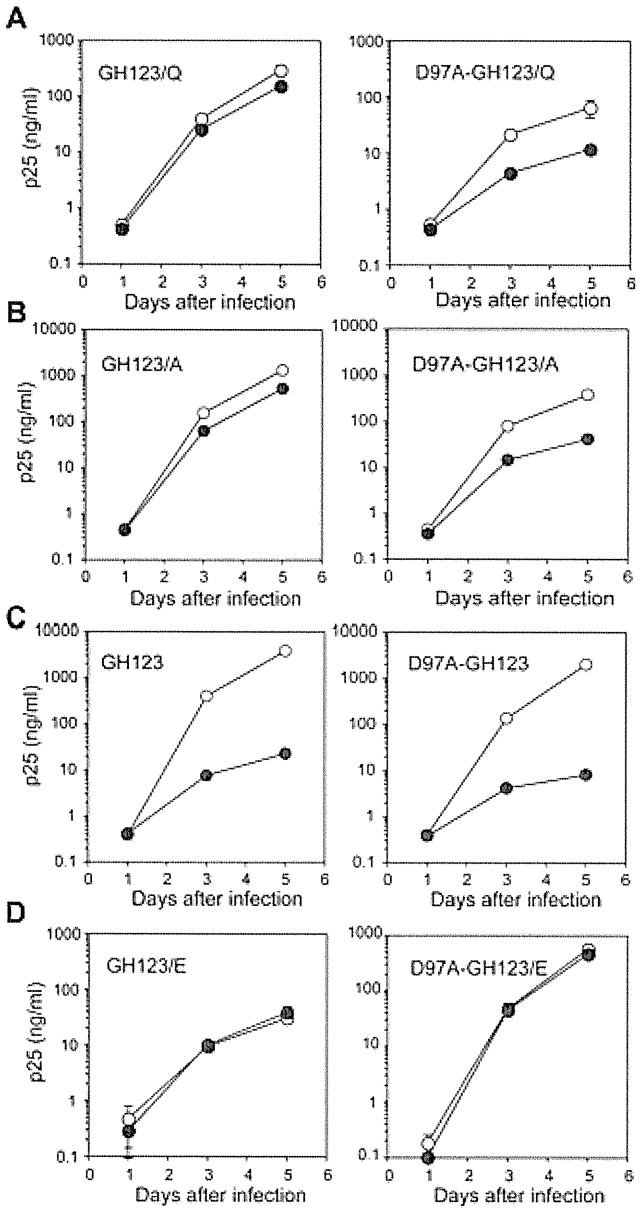

Figure 7. Effects of an aspartic acid-to-alanine substitution at the 97th position of the HIV-2 CA on viral growth in the presence or absence of CM TRIM5α. MT4 cells were infected with CM-TRIM5α-SeV (black circles) or CM-SPRY(–)-SeV (white circles) then superinfected with GH123 mutant viruses. Culture supernatants were periodically assayed for levels of viral capsid. Error bars show actual fluctuations between measurements of capsid in duplicate samples. A representative of three independent experiments is shown.
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is possible that the presence of the negative charge at the 120th position prevented access of TRIM5α even though the L4/5 conformation was adequate for TRIM5α recognition. If our modeling of GH123/E L4/5 was correct, disruption of the hydrogen bond between the 97th D and 119th R would have little or no effect on the TRIM5α sensitivity of GH123/E. In fact, the D97A substitution failed to alter the resistant phenotype of GH123/E (Figure 7D), but did unexpectedly compensate the impaired replication of GH123/E (Figure 1B). These results indicate that the effect of D97A substitution depended upon the amino acid residue at the 120th position, and further supported the notion that the L6/7 itself was also involved in CM TRIM5α restriction. Consistent with this, the side chains of amino acid residues at the 120th position were exposed on the surface of the CA (Figure 8).

When these results are considered together, it is likely that the hydrogen bond between the L4/5 and L6/7 modulates the overall structure of the exposed surface of the CA and that both L4/5 and L6/7 are responsible for CA recognition by CM TRIM5α.

Discussion

In the present study, we showed that a hydrogen bond between the 97th D and the 119th R of HIV-2 CA affected viral sensitivity to CM TRIM5α. TRIM5α-sensitive viruses showed a common L4/5 structure, but L6/7 was also important in CA recognition by TRIM5α.

Previously, we proposed that the configuration of HIV-2 CA L6/7 would affect viral sensitivity to CM TRIM5α on the basis of the results of homology modeling of the HIV-2 CA in which the 3-D structure of HIV-1 CA was used as a template [20]. In the present study, however, we performed intensive mutational analysis of the HIV-2 CA followed by more intensive computer-assisted structural analyses using the recently published 3-D structure of the HIV-2 CA and MD simulation, which provide information on structural dynamics of proteins in solution. Results of the present study revealed that alterations in the L4/5 conformation were more strongly associated with viral sensitivity to TRIM5α than those in the L6/7 configuration. Furthermore, the data on the MD simulation study disclosed that a hydrogen bond between the 97th D and the 119th R may be a critical modulator affecting the conformation of L4/5.

In the case of the HIV-1 CA, two hydrogen bonds were reported to form between R at the 229th position of Gag (R229) and E at the 245th position (E245), and between R229 and W at the 249th position (W249) [24]. These three amino acids were also found in the HIV-2 CA; and R229, E245 and W249 of the HIV-1 CA correspond to the 96th R, 112th E and the 116th W of the HIV-2 GH123 CA, respectively (Fig. 5B). The 112th E and 116th W are in the 6th helix of the CA, and the 96th R is adjacent to the 97th D in L4/5. In our HIV-2 CA models, these two hydrogen bonds were observed with a probability of more than 99.9%, regardless of the viral sensitivity to TRIM5α. Therefore, TRIM5α-resistant viruses are likely to have three hydrogen bonds at the base of L4/5, whereas those sensitive to TRIM5α have two hydrogen bonds there. It is possible that reduced structural flexibility of the base of loop causes the upper loop structure to collapse more easily. Thus, the number of the hydrogen bonds may affect the flexibility of the base of L4/5 and the maintenance of the binding surface for TRIM5α, which is formed at least partly by L4/5. As a result, the viral sensitivity to TRIM5α changes.

In the CA sequences of HIV-2 and SIVmac in the Los Alamos Database, the 97th position was always occupied by acidic D or E, and the 119th position was always occupied by R. In the case of HIV-1 or simian immunodeficiency virus isolated from the chimpanzee (SIVcpz), however, the 119th position was occupied by variable amino acid residues, while the 97th position was always occupied by acidic D or E. It should be noted that a hydrogen bond between the 97th and 119th amino acid residues was never observed in the HIV-1 CA (data not shown). Those differences may contribute to the increased sensitivity of HIV-1 to OWM TRIM5α compared with HIV-2 strains.

Although our data showed a clear correlation between viral sensitivity to TRIM5α and the conformation of CA L4/5, there was one exception. The conformation of L4/5 in GH123/E was

almost identical to those of TRIM5α-sensitive viruses, but GH123/E was highly resistant to CM TRIM5α. Furthermore, disruption of the hydrogen bond between the 97th D and the 119th R by substitution of D97A did not alter the resistant phenotype of GH123/E at all. These results suggested that the 120th amino acid residue of the HIV-2 GH123 CA itself is also involved in CM TRIM5α sensitivity independently from the L4/5 conformation. This view was also supported by our present observation that disruption of the hydrogen bond between the 97th D and the 119th R conferred only moderate sensitivity to CM TRIM5α upon another resistant virus GH123/Q (Figure 7).

Replication of GH123/E or GH123/D was slightly impaired (Figure 1B), but this impairment was compensated by the D97A substitution in GH123/E (Figure 7D). On the other hand, replication of GH123/Q was almost comparable to that of GH123 (Figure 1A); but the D97A substitution slightly impaired its replicative capability (Figure 7A). It should be also mentioned here that the viruses with basic residues at the 120th position, GH123/R and GH123/K, scarcely grew (Figure 1B). These results suggest that certain optimum levels of charge are required at the L4/5 and L6/7 for efficient viral replication. At present, it is unclear why those charge differences affect the growth capability of the virus; but it is possible that the charge difference affects the accessibility to unknown host factor(s) involved in uncoating.

HIV-2 closely resembles SIVsm, which is thought to have entered the human population on at least eight separate occasions [19]. Almost all SIV isolates from the Los Alamos Database contain glutamine at the position corresponding to the 119th or 120th position of the HIV-2 CA in the presence of strong OWM TRIM5α pressure. After entry of SIVsm into the human population, which lacks OWM TRIM5α pressure, some viruses were presumably forced to change glutamine to proline by mutating the second nucleotide of the codon. This change may have been driven by specific immune responses against the HIV-2 CA. Similarly, alanine viruses may have evolved from the proline virus after transmission to individuals lacking such responses by changing the first nucleotide of the codon in order to become more resistant to human TRIM5α. Glycine viruses may have further evolved from the alanine virus by changing the second nucleotide of the codon. However, it is unclear why serine, histidine, threonine and leucine viruses have not been identified despite their nearly normal levels of growth. It is possible that certain human immune responses prevented their emergence.

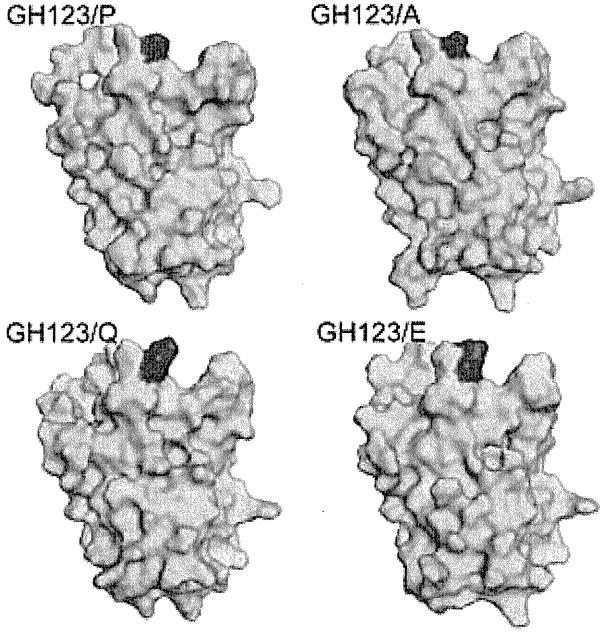

Figure 8. Surface structure of the HIV-2 capsid N-terminal domain. Surface structure of the GH123 and mutant GH123 CAs visualized with PyMOL. Red color indicates the 120th amino acid of the GH123 and mutant GH123 CAs.
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In a sharp contrast to CM TRIM5α, Rh TRIM5α could restrict both CM TRIM5α-sensitive and -resistant HIV-2 strains [8]. SIVmac239 is resistant to Rh TRIM5α, but chimeric SIVmac239 with L4/5 of HIV-2 strains GH123 [25] or ROD [21] were efficiently restricted by Rh TRIM5α. Therefore, the L4/5 of HIV-2 CA is also a critical determinant for Rh TRIM5α-mediated restriction. In the present study, we have shown that CM TRIM5α-sensitive HIV-2 viruses have a specific structure in the L4/5 of the CA. However, the 3-D structure of Rh and CM TRIM5α remains unsolved. To elucidate the more detailed molecular mechanism of the interaction between TRIM5α and the CA, structural information about TRIM5α is essential. A docking study based on such information is likely to shed light on the antiviral mechanism of TRIM5α.

In summary, we showed that a hydrogen bond between the 97th D and the 119th R of HIV-2 CA affected viral sensitivity to CM TRIM5α and that both L4/5 and L6/7 are responsible for CA recognition by CM TRIM5α.

Methods

Cell cultures

293T cells were maintained in Dulbecco's Modified Eagle medium, and HeLa cells were maintained in Minimum Essential Medium. The human T-cell line MT4 was maintained in RPMI medium. All media were supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

Plasmid construction

Mutant HIV-2 GH123 or SIVmac239 viruses were generated by site-directed mutagenesis. Infectious viruses were prepared by transfection of 293T cells with resultant proviral DNA clones. The viral titer was determined by measuring p25 or p27 with a RetroTek antigen ELISA kit (ZeptoMetrix, Buffalo, NY).

Construction of recombinant Sendai viruses (SeV) expressing C-terminally HA-tagged CM TRIM5α (CM-TRIM5α-SeV) and CM-TRIM5α lacking the PRYSPRY domain (CM-SPRY(–)-SeV) were described previously [5,20].

Viral infection

MT4 cells (1×10^5) were infected with SeV expressing each of the TRIM5αs at a multiplicity of infection of 10 plaque-forming units per cell and incubated at 37°C for 9 h. Cells were then superinfected with 20 ng of p25 of HIV-2 GH123 derivatives or with 40 ng of p27 SIVmac239 derivatives. The culture supernatants were collected periodically, and the level of p25 or p27 was measured with a RetroTek antigen ELISA kit (ZeptoMetrix).

Viral particle purification and western blotting

The culture supernatant of 293T cells transfected with plasmids encoding HIV-2 GH123 and GH123 mutants were clarified by low-speed centrifugation. The resultant supernatants (10 ml) were layered onto a 2 ml cushion of 20% sucrose and centrifuged at 35,000 rpm for 2 h at 4°C in a Beckman SW41 rotor. Pelleted viral particles were resuspended in PBS. Lysates were normalized

based on p25 antigen concentrations and were analyzed by western blotting with the SIV-infected monkey serum.

Molecular modeling and MD simulation

We used MD simulations [26] to analyze structural dynamics of the HIV-2 CA N-terminal domain. First, initial CA structures for MD simulation were constructed by homology modeling [27] using the Molecular Operating Environment, MOE 2008.1002 (Chemical Computing Group Inc., Montreal, Quebec, Canada) as described [20,28]. We used the high-resolution crystal structure of the HIV-2 CA N-terminal domain at a resolution of 1.25 Å (PDB code: 2WLV [29]) as the modeling template. Structural dynamics of these HIV-2 CA models in water environment were analyzed using MD simulations with the SANDER module in the AMBER 9 program package [30] and the AMBER99SB force field [31] with the TIP3P water model. Bond lengths involving hydrogen were constrained with SHAKE [32] and the time step for all MD simulations was set to 2 fs. After heating calculations for 20 ps to 310 K using the NVT ensemble, the simulations were executed using the NPT ensemble at 1 atm and at 310 K for 20 ns. Hydration analyses were performed using the ptraj module in AMBER. A maximum cutoff angle of 120.0° and cutoff length of 3.5 Å were used in hydrogen bond definitions. The surface structure of CA is visualized with PyMOL 1.2r1 (The PyMOL Molecular Graphics System, http://pymol.sourceforge.net/).

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Author Contributions

Conceived and designed the experiments: TS HS EEN. Performed the experiments: TM MY KK EEN. Analyzed the data: TS HS EEN. Wrote the paper: TM HS TS EEN.

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TRIM5α and species tropism of HIV/SIV

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Human immunodeficiency virus type 1 (HIV-1) infects humans and chimpanzees but not old world monkeys (OWMs) such as the rhesus monkey (Rh) and cynomolgus monkey (CM). HIV-1 efficiently enters cells of OWMs but encounters a block before reverse transcription. This narrow host range is attributed to a barrier in the host cell. In 2004, the screening of a Rh cDNA library identified tripartite motif 5α (TRIM5α) as a cellular antiviral factor. TRIM5α is one of splicing variants produced by *TRIM5* gene and TRIM5 proteins are members of the TRIM family containing RING, B-box 2, and coiled-coil domains. The RING domain is frequently found in E3 ubiquitin ligase and TRIM5α is degraded via the ubiquitin–proteasome-dependent pathway. Among TRIM5 splicing variants, TRIM5α alone has an additional C-terminal PRYSPRY (B30.2) domain. Previous studies have shown that sequence variation in variable regions of the PRYSPRY domain among different monkey species affects species-specific retrovirus infection, while amino acid sequence differences in the viral capsid protein determine viral sensitivity to restriction. TRIM5α recognizes the multimerized capsid proteins (viral core) of an incoming virus by its PRYSPRY domain and is thus believed to control retroviral infection. There are significant intraspecies variations in the Rh-*TRIM5* gene. It has also been reported that some Rh and CM individuals have retrotransposed cyclophilin A open reading frame in the *TRIM5* gene, which produces TRIM5–cyclophilin A fusion protein (TRIMCyp). TRIMCyp, which was originally identified as an anti-HIV-1 factor of New World owl monkeys, is an interesting example of the gain of a new function by retrotransposition. As different *TRIM5* genotypes of Rh showed different levels of simian immunodeficiency virus replication *in vivo*, the *TRIM5* genotyping is thought to be important in acquired immunodeficiency syndrome monkey models.

Keywords: TRIM5α, TRIMCyp, HIV-1, HIV-2, SIV, rhesus monkey, cynomolgus monkey

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is a causative agent of acquired immunodeficiency syndrome (AIDS). More than two million people are infected with HIV-1 annually around the world. Nevertheless, the host range of HIV-1 is extremely narrow, being limited to humans and chimpanzees (Gao et al., 1999). This narrow host range has hampered the establishment improved animal models of HIV-1 infection that are needed to facilitate the development of an efficacious vaccine against HIV-1 infection. In this review, we summarize current understanding regarding the species barrier of HIV-1 as discussed from the viewpoint of animal model development, focusing on tripartite motif 5α (TRIM5α), a restriction factor in monkeys.

LIFE CYCLE OF HIV-1

Human immunodeficiency virus type 1 belongs to the family Retroviridae, subfamily *Lentivirus*. It is an enveloped virus with a single-stranded RNA genome with positive polarity. HIV-1 enters $CD4^+$ T cells and macrophages through plasma membrane fusion. The virus RNA genome is subsequently reverse transcribed by viral-associated reverse transcriptase and resultant double-strand cDNA is transported to the nucleus. In the nucleus, viral-associated integrase (IN) inserts viral cDNA into the human chromosome. The transcription is enhanced by cellular activation and mRNA and full-length viral genome RNA are exported from the nucleus. Viral proteins assemble beneath the plasma membrane and virus particles bud from plasma membrane (**Figure 1**).

HOST FACTORS REQUIRED FOR HIV-1 REPLICATION IN HUMAN AND SPECIES-SPECIFIC BARRIER OF HIV-1 IN MICE

Many trials of small animal models for HIV-1 infection have failed due to lack of host factors in mice, which are necessary for efficient virus replication (**Figure 1**). CD4, the cellular receptor for HIV-1, was first identified as a host range barrier because mouse CD4 does not bind to HIV-1 envelope protein (Landau et al., 1988). Human CD4 transgenic mice, however, were not susceptible to HIV-1 infection (Lores et al., 1992). Chemokine receptors were identified as entry co-receptors (Alkhatib et al., 1996; Bleul et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996), but mice transgenic for human CD4 and either human CXCR4 (Sawada et al., 1998) or CCR5 (Browning et al., 1997) failed to show productive infection, even though murine CXCR4 is a functional co-receptor for CXCR4-tropic HIV-1 (Bieniasz et al., 1997).

Human immunodeficiency virus type 1 pre-integration complex (PIC) containing viral cDNA and viral IN are translocated into the nucleus. Two host cellular proteins have recently been

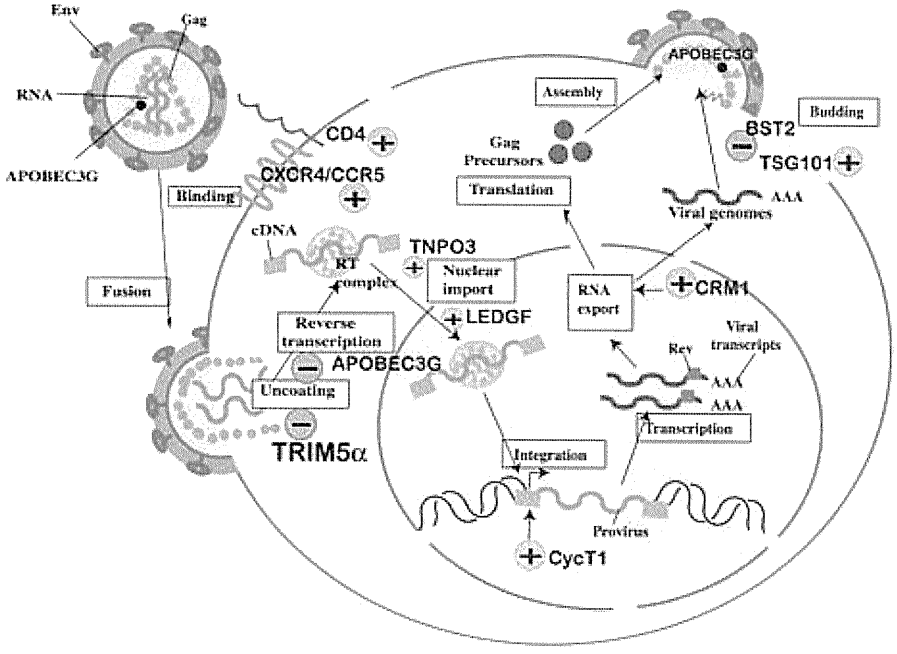

FIGURE 1 | Cellular factors involved in human immunodeficiency virus (HIV) replication cycle. (+) Positive factors required for viral replication. (–) Negative factors that suppress viral replication.

reported to mediate PIC import. The first is a lens epithelium-derived growth factor (LEDGF/p75; Cherepanov et al., 2003; Maertens et al., 2003), a protein implicated in the regulation of gene expression and cellular stress responses. LEDGF interacts with HIV-1 IN and is thought to guide PIC toward sites of active transcription for integration of viral cDNA into the human chromosome. The second is Transportin 3 (TNPO3/Transportin-SR2) identified by two independent screenings of host factors involved in HIV-1 replication (Brass et al., 2008; Christ et al., 2008). TNPO3 also binds to IN, but it is also thought to associate with viral capsid (CA) protein and supports nuclear translocation of PICs. It is currently unclear whether mouse orthologs of either or both of these factors are defective in nuclear transport and integration of HIV-1.

The integrated HIV-1 genome is then transcribed from its promoter in the long terminal repeat (LTR) by using NF-κB and Sp1 (Jones et al., 1986; Staal et al., 1990; Cullen, 1991). HIV-1 non-structural proteins, Tat, Rev, and Nef, are early gene products produced from multiply spliced mRNAs (Feinberg et al., 1986). Tat binds to the 5′ region of nascent HIV-1 transcripts and facilitates the elongation of transcribed RNA (Laspia et al., 1989; Feinberg et al., 1991). Mouse cells do not show Tat-dependent transcriptional activation of HIV-1. Cyclin T1 (CycT1) is responsible for this transcriptional barrier in mice (Newstein et al., 1990; Garber and Jones, 1999). CycT1 protein is a component of the CDK9/pTEFb transcription factor complex (Mancebo et al., 1997; Wei et al., 1998). Human but not mouse CycT1 binds to Tat and activates transcription from HIV-1 LTR (Garber et al., 1998). Nevertheless, triple-transduction of mouse cells with human CD4, CXCR4, and CycT1 was insufficient to induce productive viral infection (Bieniasz and Cullen, 2000). Additional barriers have been reported in the late stages of the viral life cycle (Mariani et al., 2000; Keppler et al., 2001; Koito et al., 2003a,b; Nagai-Fukataki et al., 2011). CRM1, a nuclear export factor that functions in association with Rev, has been suggested to be one of the late-phase factors important for the export of unspliced full-length viral genome from the nucleus (Zheng et al., 2003). Further studies are necessary to identify host cellular factors that are necessary for virus replication in humans but defective in mouse cells in order to establish small animal models of HIV-1 infection.

TRIM5α, ONE OF THE SPECIES-SPECIFIC BARRIERS TO HIV-1 IN MONKEYS

Amino acid sequences of CD4, CXCR4, CCR5, CycT1, and CRM1 in Old World monkeys (OWMs) are almost identical to those of the human orthologs, while New World monkeys, such as common marmosets and squirrel monkeys, have less functional CD4 and CCR5 receptors (LaBonte et al., 2002). Nevertheless, HIV-1 fails to replicate in activated $CD4^+$ T lymphocytes obtained from OWM, such as the rhesus monkey (Rh; *Macaca mulatta*; Shibata et al., 1995; Himathongkham and Luciw, 1996) and cynomolgus monkey (CM; *Macaca fascicularis*; Akari et al., 1996, 1999). Several studies have suggested that the blockade of HIV-1 replication in OWM cells occurs at a post-entry step (Shibata et al., 1995; Himathongkham and Luciw, 1996; Chackerian et al., 1997) and appears to result from a failure to initiate reverse transcription (Himathongkham and Luciw, 1996). Importantly, resistance against HIV-1 infection was shown to be dominant in heterokaryons between human and OWM cells, suggesting the

presence of inhibitory factor(s) against HIV-1 infection but not for simian immunodeficiency virus (SIV) in OWM cells (Munk et al., 2002).

In 2004, the screening of an Rh cDNA library identified TRIM5α as a factor that confers resistance to HIV-1 infection (Stremlau et al., 2004; **Figures 1** and **2**). Both Rh and CM TRIM5α restrict HIV-1 infection but fail to restrict SIV isolated from a macaque monkey (SIVmac; Stremlau et al., 2004; Nakayama and Shioda, 2010). In contrast, human TRIM5α is almost powerless to restrict the aforementioned viruses, but potently restricts N-tropic murine leukemia viruses (N-MLV) and equine infectious anemia virus (EIAV; Hatziioannou et al., 2004; **Figure 3**).

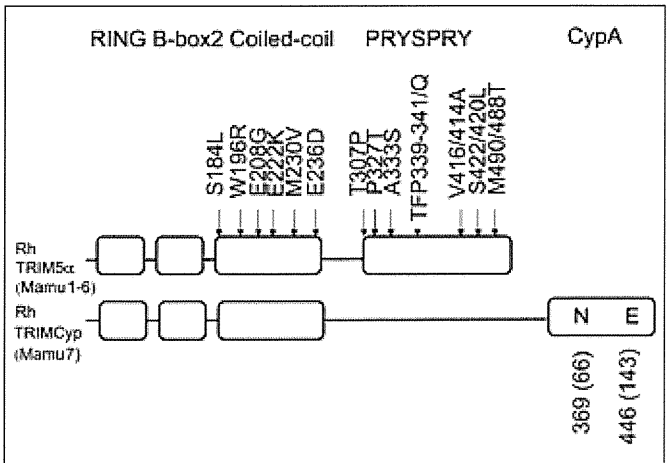

FIGURE 2 | Domains of rhesus monkey (Rh) TRIM5α and TRIMCyp proteins. The RING, B-box2, Coiled-coil, PRYSPRY, or CypA domains of Rh-TRIM5α and TRIMCyp are shown by squares. Polymorphisms are shown outside the squares. The numbers in parentheses show the amino acid positions counting from the initiation methionine codon of the CypA open reading frame.

	TRIM5α mediated viral restriction				
TRIM5α	HIV-1	SIVmac	SIVagm	N-MLV	B-MLV
Human	No	No	No	Yes	No
Rhesus monkey	Yes	No	Yes	Weak	No
Cynomolgus monkey	Yes	No	N.D.	N.D.	N.D.
AGM (tantalus)	Yes	Yes	No	Yes	No
AGM (pygerythrus)	Yes	No	No	Yes	No
Squirrel monkey	No	Yes	Weak	No	No
Owl monkey (TRIMCyp)	Yes	No	N.D.	No	No

FIGURE 3 | Species-specific restriction by TRIM5α. "Yes" denotes restriction. "Weak" denotes weak restriction. "No" denotes no restriction. "N. D." denotes no result has yet been published. SIVmac, simian immunodeficiency virus isolated from a macaque (Ohkura et al., 2006). SIVagm, simian immunodeficiency virus isolated from an African green monkey (Song et al., 2005b). N-MLV, N-tropic murine leukemia virus (Ohkura et al., 2006); B-MLV, B-tropic murine leukemia virus (Ohkura et al., 2006). AGM, African green monkey (Nakayama et al., 2005; Kim et al., 2011). Rhesus monkey (Stremlau et al., 2004; Ylinen et al., 2005; Ohkura et al., 2006), cynomolgus monkey (Nakayama et al., 2005), and owl monkey TRIMCyp (Nisole et al., 2004; Sayah et al., 2004) are also included.

TRIPARTITE MOTIF OF TRIM5α

TRIM5α is a member of the TRIM family of proteins, and consists of RING, B-box 2, coiled-coil, and PRYSPRY (B30.2) domains (Reymond et al., 2001; **Figure 2**). Proteins with the RING domains possess E3 ubiquitin ligase activity (Jackson et al., 2000); therefore, TRIM5α was thought to restrict HIV-1 by proteasome-dependent pathways. However, a proteasome inhibitor MG132 did not rescue HIV-1 infection from TRIM5α-mediated restriction, even though the levels of HIV-1 late reverse-transcription products were recovered (Anderson et al., 2006; Wu et al., 2006; Maegawa et al., 2010). TRIM5α is thus thought to use both proteasome-dependent and -independent pathways to restrict HIV-1. The distinct molecular mechanism of the proteasome-independent pathway has yet to be elucidated. It was shown that incubation of an artificially constructed HIV-1 core structure composed of the capsid–nucleocapsid (CA–NC) fusion protein with the chimeric protein containing the Rh-TRIM5α B-box 2, coiled-coil, and PRYSPRY domains and the RING domain of TRIM21 (TRIM5-21R) caused apparent breaks in the CA structure without any other cellular components (Langelier et al., 2008; Zhao et al., 2011). It is therefore likely that direct binding of Rh-TRIM5α proteins to incoming HIV-1 CA proteins causes CA disassembly, which is observed as proteasome-independent restriction.

The intact B-box 2 domain is also required for TRIM5α-mediated antiviral activity, as TRIM5α restrictive activity is diminished by several amino acid substitutions in the B-box 2 domain (Javanbakht et al., 2005). TRIM5α has been shown to form a dimer (Kar et al., 2008; Langelier et al., 2008), while the B-box 2 domain mediates higher-order self-association of Rh-TRIM5α oligomers (Li and Sodroski, 2008; Diaz-Griffero et al., 2009). The coiled-coil domain of TRIM5α is important for the formation of homo-oligomers (Mische et al., 2005), and the homo-oligomerization of TRIM5α is essential for antiviral activity (Javanbakht et al., 2006; Nakayama et al., 2006).

PRYSPRY DOMAIN OF TRIM5α, A DETERMINANT OF SPECIES-SPECIFIC RESTRICTION OF VIRUSES

The PRYSPRY domain is specific for the α-isoform among at least three splicing variants transcribed from the *TRIM5* gene. Soon after the identification of TRIM5α as a restriction factor of Rh, several studies found that differences in the amino acid sequences of the variable region 1 (V1) of TRIM5α PRYSPRY domain of different monkey species affect the species-specific restriction of retrovirus infection (Nakayama et al., 2005; Perez-Caballero et al., 2005; Sawyer et al., 2005; Stremlau et al., 2005; Yap et al., 2005; Ohkura et al., 2006; Perron et al., 2006; Kono et al., 2008, 2009). The PPYSPRY domain is thought to recognize viral cores, as TRIM5α lacking this domain does not show antiviral activity. Overexpression of truncated TRIM5α lacking the PRYSPRY domain shows a dominant negative effect on antiviral activity of full-length TRIM5α (Berthoux et al., 2005; Nakayama et al., 2006). Biochemical studies have shown that TRIM5α associates with CA in detergent-stripped N-MLV virions (Sebastian and Luban, 2005) or with an artificially constituted HIV-1 core structure composed of the CA–NC fusion protein in a PRYSPRY domain-dependent manner (Stremlau et al., 2006). Although the precise three-dimensional crystal structure of the

PRYSPRY domain has not been resolved, TRIM5-21R assembled and formed two-dimensional paracrystalline hexagonal arrays *in vitro* (Ganser-Pornillos et al., 2011). This assembly required RING and B-box 2 domains but was independent of the PRYSPRY domain. However, the hexagonal lattices of HIV-1 CA that mimic the surface of core act as template for stabilization of TRIM5-21R arrays in a PRYSPRY-dependent manner (Ganser-Pornillos et al., 2011). As the interaction between individual CA monomers and TRIM5α is very weak, CA recognition by TRIM5α is thought to be a synergistic combination of direct binding interactions with the PRYSPRY domain, higher-order assembly of TRIM5α, template-based assembly, and lattice complementarity.

VARIABLE SUSCEPTIBILITY OF SIMIAN IMMUNODEFICIENCY VIRUSES AMONG MONKEY SPECIES

Simian immunodeficiency virus isolated from sooty mangabey (SIVsm) and SIV isolated from African green monkey (SIVagm) replicate in their natural hosts (VandeWoude and Apetrei, 2006) and $CD4^+$ human cells. SIVmac evolved from SIVsm in captive macaques, and replicates efficiently in Rh (Shibata et al., 1995; Himathongkham and Luciw, 1996) and CM (Akari et al., 1996, 1999) as well as in human $CD4^+$ cells but not in African green monkey (AGM) cells. We found that a 37-amino acid residue region including a 20-amino acid duplication in the V1 of AGM TRIM5α determined species-specific restriction against SIVmac239 (Nakayama et al., 2005). However, AGM TRIM5α failed to restrict SIVagm, which naturally infects AGM, while Rh-TRIM5α can restrict SIVagm infection (Song et al., 2005b; **Figure 3**).

In contrast to HIV-1, AGM TRIM5α restricted SIVmac239 mainly in a proteasome-dependent manner, as SIVmac239 escaped completely from attacks by RING mutants of TRIM5α that could still moderately restrict HIV-1 infection. Kim et al. reported that AGM TRIM5α derived from *Chlorocebus tantalus* but not *Chlorocebus pygerythrus* subspecies of AGM restrict SIVmac239, while both potently restrict HIV-1 (**Figure 3**). Both AGM TRIM5α share the 20-amino acid duplication but a *C. pygerythrus*-specific leucine residue at the 34th position within the RING domain compromised the ability of *C. pygerythrus* AGM TRIM5α to restrict SIVmac239 infection (Kim et al., 2011). This result is consistent with the observation of RING-proteasome dependency of SIVmac239 restriction by TRIM5α.

Human immunodeficiency virus type 2 (HIV-2) is assumed to have originated from SIVsm as a result of zoonotic events involving monkeys and humans (Hahn et al., 2000). Previous studies have shown that HIV-2 strains vary widely in their ability to grow in cells of OWM, such as baboons, Rh, and CM (Castro et al., 1990, 1991; Locher et al., 1998, 2003; Fujita et al., 2003). By testing CM and Rh recombinant TRIM5α, three amino acid residues of TFP at the 339th to 341st positions of Rh-TRIM5α V1 were shown to be indispensable for restricting particular HIV-2 strains that are still resistant to CM TRIM5α bearing a single Q instead of TFP at the 339th to 341st positions (Kono et al., 2008; **Figure 4**). The TFP motif is also critical to restrict SIVsm (Kirmaier et al., 2010). Baboon and sooty mangabey (SM) TRIM5α bearing SFP at the 339th to 341st positions can potently restrict HIV-1, only weakly restrict HIV-2, and failed to restrict SIVmac239 (Newman et al., 2006; Kono et al., 2008, 2009).

VIRAL DETERMINANT OF SENSITIVITY TO MONKEY TRIM5α

Tripartite motif 5α is thought to recognize viral cores through its PRYSPRY domain. To determine the region in viral CA that interacts with TRIM5α, we focused on HIV-2, which closely resembles SIVmac (Hahn et al., 2000). Sequence analysis showed that the CM TRIM5α-sensitive viruses had proline (P) at the 119th or

		TRIM5α/TRIMCyp allels					
Viruses	Partial capsid sequences	Rh^{TFP}	Rh^{Q}	CM	Rh^{CypA}	$CM^{CypA(NE)}$	$CM^{CypA(DK)}$
HIV2 GH123	GP**LPA**GQLRDP**R**GSDIAGTTSTVEEQIQWMYR**P**	Yes	Yes	Yes	Yes	Yes	No
HIV2 UC1	GP**LPA**GQLRDP**R**GSDIAGTTSTVEEQIQWMYR**A**	Yes	No	No	Yes	Yes	No
HIV2 UC2	GP**LPA**GQLRDP**R**GSDIAGTTSTVDEQIQWMYR**Q**	Yes	No	No	Yes	Yes	No
SIVsmE543	GP**LPA**GQLREP**R**GSDIAGTTSTVEEQIQWMYR**Q**	Yes	No	No	Yes	Yes	N.D.
SIVsmE041	GP**IPA**GQLREP**R**GSDIAGTTSTVEEQIQWMYR**Q**	Yes	No	No	Yes	Yes	N.D.
SIVmac239	AP**QQ**-GQLREP**S**GSDIAGTTSSVDEQIQWMYR**Q**	No	No	No	No	No	No
SIVmac251	AP**QQ**-GQLREP**S**GSDIAGTTSSVDEQIQWMYR**Q**	No	No	No	No	No	No
HIV1 NL4-3	GP**IAP**GQMREP**R**GSDIAGTTSTLQEQIGWMT-**H**	Yes	Yes	Yes	No	No	Yes

FIGURE 4 | HIV-2/SIV capsid sequence variations and restriction patterns of rhesus (Rh) and cynomolgus monkey (CM) TRIM5α/TRIMCyp alleles. "Yes" denotes restriction. "Weak" denotes weak restriction. "No" denotes no restriction. "N. D." denotes no result has yet been published. The unique QQ sequence at the 89th–90th positions of SIVmac, which is critical for escape from Rh TRIMCyp, Rh^{CypA} (Kirmaier et al., 2010), is shown in red. Arginine 97 at the base of the loop between helices 4 and 5, which is important to escape from TFP alleles of Rh-TRIM5α, Rh^{TFP} (Kirmaier et al., 2010), is shown in blue. The glutamine and alanine residues at position 120 of GH123 or analogous positions of other HIV-2 strains, which is critical for resistance against Q alleles of Rh-TRIM5α, Rh^{Q} (Kirmaier et al., 2010) and CM TRM5α (Song et al., 2007; Kono et al., 2008), are shown in green. $CM^{CypA(NE)}$ and $CM^{CypA(DK)}$ denote the minor and major alleles of CM TRIMCyp, respectively.

120th position of CA, while the CM TRIM5α-resistant viruses had alanine (A), glutamine (Q), or glycine (G) at the same position (**Figure 4**). Replacing the proline of a CM TRIM5α-sensitive HIV-2 molecular clone with A, Q, or G changed the phenotype from sensitive to resistant and the mutant viruses replicated well in the presence of CM TRIM5α. The reverse was observed when the glutamine of a resistant SIVmac molecular clone was replaced with proline (Song et al., 2007; Miyamoto et al., 2011). The 119th or 120th position is located in a loop between α-helices 6 and 7 (L6/7; **Figure 5**).

In the case of Rh-TRIM5α, Ylinen et al. replaced a loop between α-helices 4 and 5 (L4/5) of SIVmac239 CA with that of HIV-2 in the SIVmac239 background and found that the resultant mutant virus showed impaired growth ability in Rh cells compared with the parental SIVmac239. However, the reciprocal virus with SIVmac239 CA L4/5 in the HIV-2 background did not gain resistance against Rh-TRIM5α, suggesting that Rh-TRIM5α interacts mainly with L4/5 but other portion(s) of HIV-2 CA are also involved (Ylinen et al., 2005). Lin and Emerman (2008) also reported that SIVagm with HIV-1 L4/5 and L6/7 was susceptible to Rh-TRIM5α restriction. In fact, we found that the 120th amino acid of HIV-2 CA, the determinant of CM TRIM5α sensitivity, also contributes to Rh-TRIM5α susceptibility (Kono et al., 2010). Furthermore, studies on chimeric viruses between Rh-TRIM5α-sensitive HIV-2 and Rh-TRIM5α-resistant SIVmac239 revealed that multiple regions including L4/5 in the N-terminal half of SIVmac239 CA contribute to evasion of SIVmac239 from Rh-TRIM5α (Kono et al., 2010; **Figure 5**).

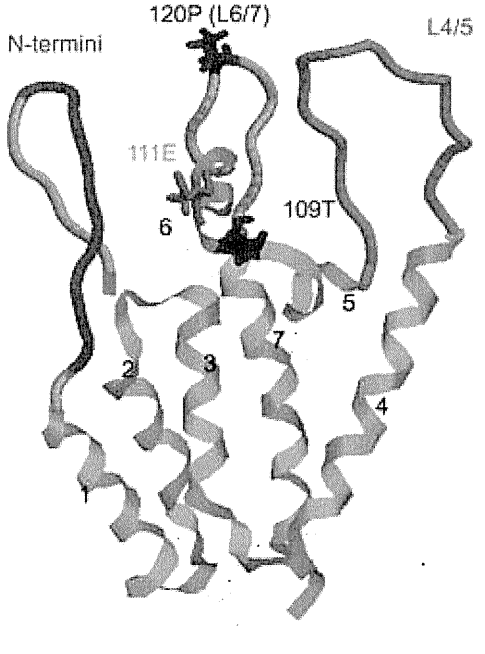

FIGURE 5 | Structure of the N-terminal half of HIV-2 capsid monomer. The ribbons represent the backbone of HIV-2 capsid proteins, and seven α-helices are labeled. The positions important in Rh-TRIM5α recognition are highlighted as N-termini (the 5th to 13th amino acid residues) in purple, the loop between α-helices 4 and 5 (L4/5) in green, the 109th T in blue, the 111th E in orange, and the 120th P in red.

To elucidate further details regarding the structure of CA recognized by TRIM5α, we generated mutant HIV-2 viruses each carrying 1 of 20 amino acid residues at position 120, and examined their susceptibilities to CM TRIM5α-mediated restriction. Amino acid residues with hydrophobic side chains or aromatic rings were associated with sensitivity to CM TRIM5α, while those with small side chains or amide groups conferred resistance (Miyamoto et al., 2011). Computer-assisted three-dimensional models showed that the mutations at the 120th position in L6/7 affected the conformation of the neighboring loop L4/5 by a hydrogen bond between aspartic acid 97 in L4/5 and arginine 119 in L6/7 (**Figure 6**).

Taken together, these observations suggested that TRIM5α recognized the overall outer surface of the N-terminal half of viral CA including L4/5 and L6/7. To determine further details regarding the interaction between CA and TRIM5α, biochemical and structural analyses of the PRYSPRY domain, especially the V1 loop bound with CA, are required. In contrast to SIV/HIV-2, the L4/5 loop of HIV-1 also binds cyclophilin A (CypA). It is not yet clear whether monkey TRIM5α does or does not recognize HIV-1 CA with endogenous CypA.

INTRASPECIES GENETIC VARIATION OF THE Rh-*TRIM5* GENE

The *TRIM5* gene varies considerably among primate species (Sawyer et al., 2005; Song et al., 2005a; Newman et al., 2006). It is not surprising that the PRYSPRY domain is highly variable as TRIM5α interacts with the retroviral core through this region, as described above, and the main pressure for positive selection may be endogenous retroviruses (Kaiser et al., 2007). Interestingly, there is a 339TFP341-to-Q polymorphism in Rh-TRIM5α (Newman et al., 2006; **Figure 2**), which reduces its anti-HIV-2 activity (Kono et al., 2008). Newman et al. (2006) grouped Rh-TRIM5α into six alleles (*Mamu-1* to *-6*) including rare alleles *Mamu-2* and *Mamu-6*. Wilson et al. (2008a) showed that *Mamu-1* and *-3* alleles restrict HIV-1, HIV-2, EIAV, and feline immunodeficiency virus (FIV), but not N-MLV, B-MLV, or SIVmac239, while *Mamu-4* and *-5* alleles restrict HIV-1, EIAV, and FIV but not HIV-2, N-MLV, B-MLV, or SIVmac239 using a TRIM5α-transduced cat cell line (CRFK).

Lim et al. independently reported 11 Rh-TRIM5α alleles in which alleles 1–5 contained 339TFP341. Remaining alleles 6–11 contained 339Q. They established B-lymphoblastoid cell lines (B-LCLs) from Rh and used these B-LCLs for infection with VSV-G pseudo-typed GFP-expressing viruses. They found more GFP-positive cells in B-LCLs with Rh-TRIM5α Q allele than in B-LCLs with Rh TFP allele infected with SIVmac239-, HIV-1-, and SIVsmE543-based GFP-expressing viruses. It should be noted that the anti-HIV-1 activity of the Rh-TRIM5α Q allele is significantly stronger than the anti-SIVmac239 and SIVsmE543 activities of the Rh-TRIM5α TFP allele (Lim et al., 2010b). Lim et al. (2010a,b) retrospectively analyzed plasma viral load of Rh after SIVmac251 challenge by intravenous route and found that Rh with the Q allele was associated with higher levels of plasma viral RNA at the time when the levels of viral RNA stabilized after a period of acute infection (0.6 log median difference at 70 days after infection), more rapid loss of central memory $CD4^+$ T cells, and higher rate of progression to AIDS. These results were consistent with their own *in vitro* observations described above.

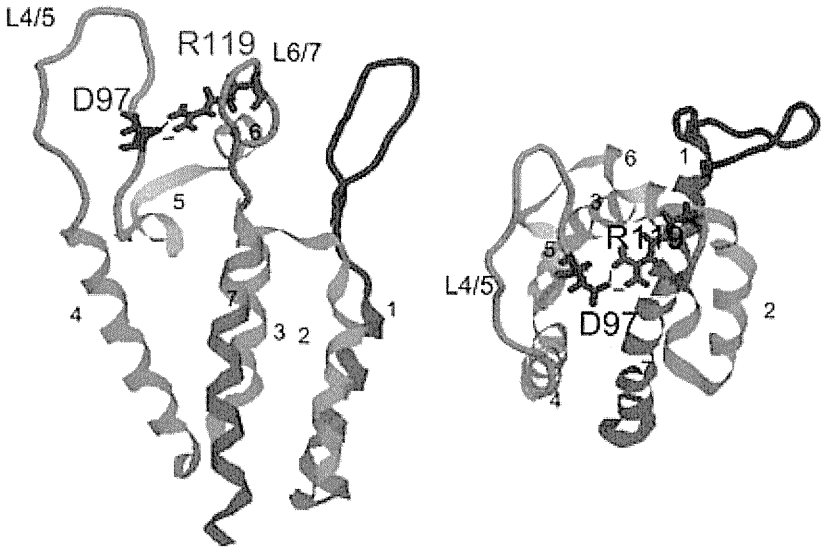

FIGURE 6 | The hydrogen bond between two external loops of HIV-2 capsid. The structures of the N-terminal domain of GH123 are shown, and seven color-coded α-helices are labeled. Blue and red wireframes denote side chains of glutamic acid at the 97th (D97) and arginine at the 119th (R119) positions, respectively. Black lines indicate hydrogen bonds between D97 and R119. Models are shown from two different angles.

On the other hand, Wilson et al. (2008a) failed to detect anti-SIVmac239 activity of both Rh-TRIM5α Q and TFP alleles. Similarly, Kirmaier et al. (2010) detected virtually no anti-SIVmac239 activity in both Rh-TRIM5α TFP and Q alleles (**Figure 4**), although numbers of infected cells in *Mamu-4* (Rh-TRIM5α Q allele) are slightly higher than those in *Mamu-1* (Rh-TRIM5α TFP allele). In contrast, Kirmaier et al. (2010) reported that the Rh-TRIM5α TFP allele restricted SIVsmE543 and SIVsmE041, although the Rh-TRIM5α Q allele did not show any anti-SIVsmE543 or anti-SIVsmE041 activity. SIVmac239 is a molecular clone of a highly adapted, emergent virus of Rh, generated in the 1980s by experimental passage of SIV-positive plasma through several monkeys (Daniel et al., 1985). In contrast, SIVsmE041 is a primary isolate from SM and SIVsmE543 was cloned after experimental passage of SIVsm through two Rh (Hirsch et al., 1997). SIVmac and SIVsm shared Q at the 118th position of CA, corresponding to the 120th position of GH123 (HIV-2), but SIVmac239 and SIVmac251 have an R-to-S change at position 97 at the base of L4/5 of CA that are critical for resistance against Rh-TRIM5α TFP allele (**Figure 4**).

In the case of SIVsmE543 *in vivo*, Rh-TRIM5α$^{TFP/TFP}$ homozygotes markedly diminished viral replication compared to Rh-TRIM5α$^{Q/Q}$ homozygotes at peak (2 log reduction) and 8 weeks (3 log reduction) after intravenous or intrarectal infection, consistent with the *in vitro* results (Kirmaier et al., 2010). It should be noted that the suppression of SIVsmE543 by Rh-TRIM5α TFP is more dramatic than that of SIVmac251. In low-dose repeated mucosal challenge experiments, two groups reported similar results using SIVsmE660, the CA sequence of which closely resembles that of SIVsmE543 (Reynolds et al., 2011; Yeh et al., 2011). Several studies evaluated MHC class I and *TRIM5* genotypes in SIV-infected Rh, and concluded that *TRIM5* genotype independently affected plasma viral load and survival rate after SIV infection (Lim et al., 2010a; Reynolds et al., 2011; Yeh et al., 2011). Taken together, these observations indicate that it is necessary to perform *TRIM5* genotyping of Rh when using SIVsm. It is also better to do so when using SIVmac239 and SIVmac251, although Fenizia et al. (2011) recently reported that there was no difference in SIVmac251 susceptibility among Rh with different TRIM5 genotypes in repeated rectal challenge.

TRIM5 AND CypA FUSION PROTEIN IN NEW WORLD MONKEY

Cells of the NWM, owl monkey (*Aotus trivirgatus*), are resistant to HIV-1 infection. Treatment of owl monkey cells with cyclosporin A, an inhibitor of CypA, allowed HIV-1 infection (Towers et al., 2003). In 2004, soon after the discovery of TRIM5α, analysis of the owl monkey *TRIM5* gene identified a long interspersed nuclear element (LINE)-1-mediated retrotransposition of CypA between exons 7 and 8, resulting in expression of a fusion protein designated as TRIMCyp (Nisole et al., 2004; Sayah et al., 2004). Owl monkey TRIMCyp contained the N-terminal half of TRIM5α, RING, B-box 2, and coiled-coil, but the PRYSPRY domain was replaced with CypA. As the CypA domain of owl monkey TRIMCyp binds to L4/5 of HIV-1 CA, owl monkey TRIMCyp showed similar antiviral activity to TRIM5α (**Figure 3**). The interaction between HIV-1 CA and CypA can be inhibited by cyclosporine A. This is a very interesting example of a gain-of-function by retrotransposition. The owl monkey has been shown to express only TRIMCyp, and not TRIM5α.

TRIMCyp IN OWMs

The expression of TRIMCyp was thought to be an anomaly unique to owl monkeys, but in 2008 another CypA insertion was found in several species of OWMs belonging to the Genus *Macaca*, Rh,

CM, and the pig-tailed monkey (PM; *Macaca nemestrina*; Brennan et al., 2008; Newman et al., 2008; Virgen et al., 2008; Wilson et al., 2008b). It is reasonable to assume that the retrotransposition event occurred in the common ancestor of these three macaques. Insertion of the CypA gene was at the 3′ end of the *TRIM5* gene, which is different from the owl monkey, indicating that CypA retrotransposition into the *TRIM5* gene in OWMs occurred independently from that in owl monkeys. A G-to-T transversion linked with CypA insertion altering the canonical splicing acceptor of *TRIM5* exon 7 caused alternative splicing (Brennan et al., 2008). The resultant mRNA lacks exons 7 and 8, and the PRYSPRY domain is replaced with CypA. In PM, TRIM5α mRNA is absent. Instead, TRIM5 isoforms TRIM5θ and TRIM5η were detected. These isoforms are splicing variants of the TRIMCyp (Brennan et al., 2008). TRIM5θ is truncated at the N-terminus of the PRYSPRY domain and TRIM5η lacks nine amino acid residues encoded by exon 7 (Brennan et al., 2007). PM TRIMCyp restricted HIV-2 but not HIV-1 infection (Liao et al., 2007; Brennan et al., 2008).

In Rh, the allele frequency of TRIMCyp (*Mamu-7*) was 25% in an Indian population but TRIMCyp was completely absent from a Chinese population (Wilson et al., 2008b). Rh TRIMCyp restricted HIV-2 but not HIV-1 infection (Wilson et al., 2008b). In CM, Brennan et al. (2008) initially reported that the amino acid residue at position 357 of CM TRIMCyp, corresponding to position 54 counting from the methionine of CypA, was arginine (R), and CM TRIMCyp with R at this position failed to restrict HIV-1. Subsequently, Ylinen et al. (2010) reported another allele of CM TRIMCyp encoding histidine (H) at this position (*Mafa TRIMCyp2*) and Mafa TRIMCyp2 protein potently restricted HIV-1 but not HIV-2. Recently, Dietrich et al. (2011) examined 15 CMs from Indonesia, Indochina, Mauritius, and the Philippines carrying TRIMCyp, and did not find R at this position. We also examined 64 CMs from Malaysia, the Philippines, and Indonesia carrying TRIMCyp (34 heterozygotes and 30 homozygotes for TRIMCyp), and found that none of these 94 TRIMCyp genes carried R at this position (Saito et al., 2011a). On the other hand, both Dietrich et al. and our group found that TRIMCyp frequency in CM was apparently higher than that in Rh. TRIMCyp frequency tended to be higher in eastern Asia than in western Asia. Dietrich et al. and our group also found major and minor haplotypes of CM TRIMCyp with single nucleotide polymorphisms in the CypA domain. The major haplotype of CM TRIMCyp bears aspartic acid (D) and lysine (K) at positions 369 and 446, respectively (Brennan et al., 2008; Ylinen et al., 2010). The minor haplotype encodes asparagine (N) and glutamic acid (E) at positions 369 and 446, respectively (Dietrich et al., 2011; Saito et al., 2011a). N369 and E446 were also found in PM and Rh TRIMCyps, and the CypA portion of the NE haplotype of CM TRIMCyp has the same amino acid sequence as that of Rh TRIMCyp. The major CM haplotype of the TRIMCyp suppressed HIV-1 but not HIV-2, while the minor haplotype of TRIMCyp suppressed HIV-2 but not HIV-1 as PM and Rh TRIMCyp did (Saito et al., 2011a; **Figure 4**).

The original CypA sequence retrotransposed into the macaque *TRIM5* locus must have been the authentic macaque CypA. There are two or three amino acid differences between authentic CypA and the CypA portion of TRIMCyp in Rh, CM, and PM, and TRIMCyp with the authentic CypA sequence has been shown to restrict HIV-1 but to only weakly restrict HIV-2 (Virgen et al., 2008; Price et al., 2009). TRIMCyp from all three of these OWM species share H at the 372nd position, corresponding to the 69th position of CypA where the authentic macaque CypA has R. Rh and PM TRIMCyps and the minor haplotype of CM TRIMCyp share N at the 369th position (the 66th position in CypA), where the authentic CypA and major haplotypes of CM TRIMCyp (*Mafa TRIMCyp2*) have D. Structural analysis of CypA domain revealed that these mutations caused drastic changes in configuration of the active site loop (from the 64th amino acid residue to the 74th residue in CypA) in Rh TRIMCyp, leading to a decreased binding affinity to HIV-1 CA but an increased affinity to HIV-2 CA (Price et al., 2009). Therefore, these mutations enhanced antiviral activity of TRIMCyp against HIV-2 but diminished anti-HIV-1 activity (Price et al., 2009). In the case of the major haplotype of CM TRIMCyp, an additional E-to-K change at the 446th position (the 143rd position in CypA) decreased affinity to HIV-2 CA by its positive charge (Ylinen et al., 2010), and the D at the 369th position (the 66th position in CypA) supported its anti-HIV-1 activity.

How did these interspecies and intraspecies variations occur in TRIMCyp? It is reasonable to assume that the R-to-H mutation at the 372nd position (R69H) together with the D-to-N mutation at the 369th position (D66N), which enhanced antiviral activity of TRIMCyp against HIV-2 but diminished anti-HIV-1 activity (Price et al., 2009), arose early in a macaque common ancestor. After the separation of CM from other species, an additional E-to-K change at the 446th position (E143K) and the N-to-D reversion at the 369th position (N66D) may also have occurred in the major haplotype of CM TRIMCyp. Alternatively, polymorphisms at the 369th and 446th positions may have arisen early in a macaque common ancestor but only CM could transmit these polymorphisms until the present. As described above, CM TRIM5α has Q at amino acid position 339 (Nakayama et al., 2005), where Rh-TRIM5α has a Q-to-TFP polymorphism (Newman et al., 2006; **Figure 2**). This Q-to-TFP polymorphism in the PRYSPRY domain also altered the spectrum of anti-lentiviral activity of TRIM5α (Kono et al., 2008; Wilson et al., 2008a; Kirmaier et al., 2010; Lim et al., 2010b; **Figure 4**). Therefore, it is tempting to speculate that the selection pressure in CM drove amplification and diversification in TRIMCyp, while that in Rh drove diversification of the PRYSPRY domain of TRIM5α. It will be of interest to examine what retroviruses have driven the evolution of TRIMCyp and *TRIM5* genes (**Figure 7**).

With respect to SIV infection, Rh TRIMCyp failed to restrict SIVmac239 (Brennan et al., 2008; Wilson et al., 2008b; Kirmaier et al., 2010) but could restrict SIVsm (Kirmaier et al., 2010). The unique LPA-to-QQ change at positions 89–91 in L4/5 of SIVmac was critical for escape from Rh TRIMCyp (**Figure 4**). Rh heterozygous for the TFP allele of TRIM5α and TRIMCyp suppressed viral infection of both SIVsmE543 (Kirmaier et al., 2010) and SIVsm660 (Reynolds et al., 2011) more efficiently than Rh homozygous for either TRIMCyp or TRIM5α. It is possible that Rh heterozygous for the TFP allele of TRIM5α and TRIMCyp express two different molecules that bind distinct regions of CA and eliminate incoming virus more effectively than Rh with TRIM5 molecules targeting only one region of CA.

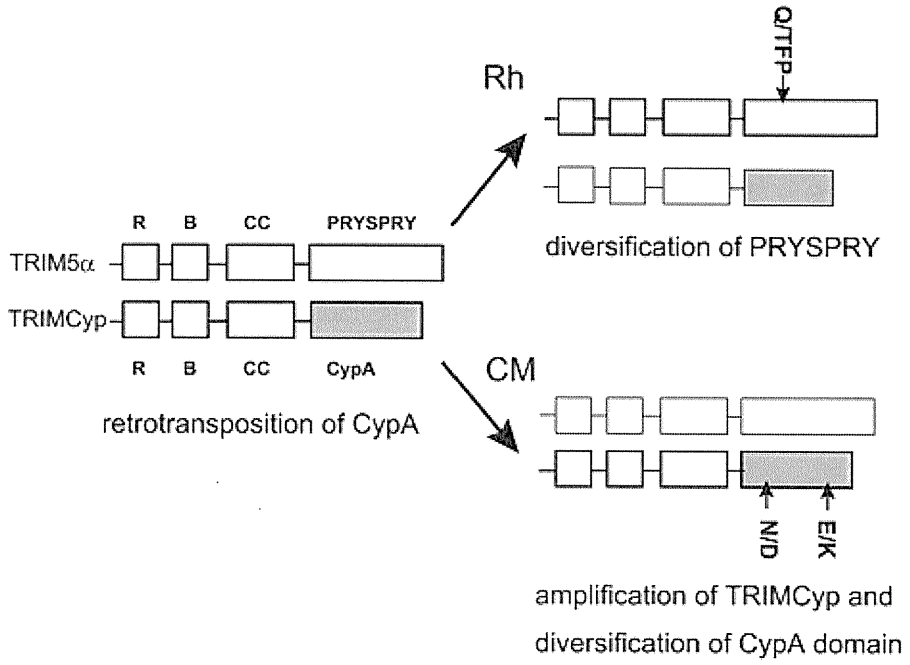

FIGURE 7 | Diversity of Rh and CM *TRIM5* genes. The RING, B-box2, Coiled-coil, and PRYSPRY domains of TRIM5α and TRIMCyp are shown by squares. CypA domains in TRIMCyp are shown as filled squares. Major alleles in Rh and CM are shown in bold lines. Polymorphisms are shown outside the squares.

OTHER RESTRICTION FACTORS AND DEVELOPMENT OF MONKEY-TROPIC HIV-1

To establish a monkey model for the study of HIV-1/AIDS, Kamada et al. (2006) developed an HIV-1 strain with minimal segments of SIVmac239. This virus (NL-ScaVR and DT5R) contains the L4/5 of CA and the entire *vif* segment of SIVmac239, and was designed to escape restriction mediated by ApoB mRNA editing catalytic subunit (APOBEC) 3G and CypA in OWM cells. APOBEC3G modifies the minus strand viral DNA during reverse transcription, resulting in impairment of viral replication (Sheehy et al., 2002; Harris et al., 2003; Mangeat et al., 2003), but this activity could be counteracted by the viral Vif protein (Mariani et al., 2003; Marin et al., 2003; Sheehy et al., 2003). Although HIV-1 Vif can potently suppress human APOBEC3G, it is not effective against Rh APOBEC3G, which at least partly explains the restriction of HIV-1 replication in monkey cells. CypA binds directly to L4/5 of HIV-1 CA but not to SIVmac CA and augments HIV-1 infection in human cells but inhibits its replication in OWM cells (Kootstra et al., 2003; Berthoux et al., 2004; Nakayama et al., 2008). Although DT5R could replicate in PM primary $CD4^+$ T cells as well as in the CM T cell line HSC-F but not in Rh cells (Kamada et al., 2006), inoculation of this monkey-tropic HIV-1 (HIV-1mt) into PM did not cause $CD4^+$ T cell depletion or any clinical symptoms (Igarashi et al., 2007), probably due to inefficient viral growth in monkeys. In the case of CM, replacement of L6/7 of HIV-1 with that of SIVmac239 greatly enhanced viral replication in PBMC (Kuroishi et al., 2009, 2010) and in animals (Saito et al., 2011b). However, the virus could not escape completely from CM TRIM5α (Kuroishi et al., 2010). Another HIV-1mt carrying 202 amino acid residues of SIVmac239 CA and *vif*, generated by Hatziioannou et al. (2006), could replicate efficiently in Rh cells, confirming that the N-terminal half of CA is required to be that of SIVmac to escape from Rh-TRIM5α. Unfortunately, this virus has replaced nearly all of CA sequence with that of SIVmac239 and has lost important CTL epitopes of HIV-1, and thus further improvement is required to use Rh as an HIV-1 infection model. H87Q and/or V86M mutations induced by adaptation of HIV-1 to the cells expressing Rh-TRIM5α (Pacheco et al., 2010) would be useful. In contrast, lack of functional TRIM5α expression in PM enabled Hatziioannou et al. (2009) to construct an HIV-1mt strain that differs from HIV-1 only in the *vif* gene and can efficiently replicate in PM. This is the most promising HIV-1/monkey model at present, if PMs are available in sufficient numbers for research.

Other host factors capable of suppressing HIV-1 replication were recently identified (**Figure 1**). One is tetherin (also know as BST2 or CD317; Neil et al., 2008; Van Damme et al., 2008). BST2 is an interferon-inducible membrane protein that interferes with the detachment of virus particles from infected cells. HIV-1 overcomes this restriction by expressing an accessory protein, Vpu, which counteracts BST2. BST2 restriction is also counteracted by primate lentiviruses that do not express a Vpu protein. Anti-BST2 functions are provided by the Env protein in HIV-2 and SIVtan (Gupta et al., 2009) or the Nef protein in SIVsm/mac and SIVagm (Jia et al., 2009; Zhang et al., 2009). As chimeric virus containing the tat, rev, vpu, and env of the HXB2 strain of HIV-1 in the genetic background of SIVmac239 is pathogenic in Rh and PM (Joag et al., 1996), BST2 in monkeys can be canceled by HIV-1 Vpu. Another recently identified host factor is SAMHD1 as dendritic and myeloid-cell-specific

HIV-1 restriction factor counteracted by HIV-2/SIV Vpx (Laguette et al., 2011; Yeh et al., 2011). As HIV-1 lacks Vpx, it is necessary to elucidate whether monkey SAMHD1 restricts HIV-1.

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Dominant induction of vaccine antigen-specific cytotoxic T lymphocyte responses after simian immunodeficiency virus challenge

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ABSTRACT

Cytotoxic T lymphocyte (CTL) responses are crucial for the control of human and simian immunodeficiency virus (HIV and SIV) replication. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. We previously developed a CTL-inducing vaccine and showed SIV control in some vaccinated rhesus macaques. These vaccine-based SIV controllers elicited vaccine antigen-specific CTL responses dominantly in the acute phase post-challenge. Here, we examined CTL responses post-challenge in those vaccinated animals that failed to control SIV replication. Unvaccinated rhesus macaques possessing the major histocompatibility complex class I haplotype *90-088-Ij* dominantly elicited SIV non-Gag antigen-specific CTL responses after SIV challenge, while those induced with Gag-specific CTL memory by prophylactic vaccination failed to control SIV replication with dominant Gag-specific CTL responses in the acute phase, indicating dominant induction of vaccine antigen-specific CTL responses post-challenge even in non-controllers. Further analysis suggested that prophylactic vaccination results in dominant induction of vaccine antigen-specific CTL responses post-viral exposure but delays SIV non-vaccine antigen-specific CTL responses. These results imply a significant influence of prophylactic vaccination on CTL immunodominance post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

1. Introduction

In human and simian immunodeficiency virus (HIV and SIV) infections, cytotoxic T lymphocyte (CTL) responses exert strong suppressive pressure on viral replication but fail to control viremia leading to AIDS progression [1–5]. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. It is important to determine how prophylactic CTL memory induction affects CTL responses in the acute phase post-viral exposure.

We previously developed a prophylactic AIDS vaccine (referred to as DNA/SeV-Gag vaccine) consisting of DNA priming followed by boosting with a recombinant Sendai virus (SeV) vector expressing SIVmac239 Gag [6]. Evaluation of this vaccine's efficacy against a SIVmac239 challenge in Burmese rhesus macaques showed that some vaccinees contained SIV replication [7]. In particular, vaccination consistently resulted in SIV control in those animals possessing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* [8]; $Gag_{206-216}$ (IINEEAADWDL) and $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses were shown to be responsible for this vaccine-based SIV control [9]. Furthermore, in a SIVmac239 challenge experiment of *90-120-Ia*-positive macaques that received a prophylactic DNA/SeV vaccine expressing the $Gag_{241-249}$ epitope fused with enhanced green fluorescent protein (EGFP), all the vaccinees controlled SIV replication [10]. This single epitope vaccination resulted in dominant $Gag_{241-249}$-specific CTL responses with delayed $Gag_{206-216}$-specific CTL induction after SIV challenge, whereas $Gag_{206-216}$-specific and

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$Gag_{241-249}$-specific CTL responses were detected equivalently in unvaccinated *90-120-Ia*-positive animals.

These previous results in vaccine-based SIV controllers indicate dominant induction of vaccine antigen-specific CTL responses post-challenge, implying that prophylactic vaccination inducing vaccine antigen-specific CTL memory may delay CTL responses specific for viral antigens other than vaccine antigens (referred to as non-vaccine antigens) post-viral exposure. In these SIV controllers, the reduction of viral loads could be involved in delay of SIV non-vaccine antigen-specific CTL responses. Then, in the present study, we examined the influence of prophylactic vaccination on immunodominance post-challenge in those vaccinees that failed to control SIV replication. Our results showed dominant induction of vaccine antigen-specific CTL responses post-challenge even in these SIV non-controllers.

2. Materials and methods

2.1. Animal experiments

The first set of experiment used samples in our previous experiments of six Burmese rhesus macaques (Macaca mulatta) possessing the MHC-I haplotype *90-088-Ij* (macaques R02-004, R02-001, and R03-015, previously reported [7,11]; R04-014, R06-022, and R04-011, unpublished). Three of them, R02-001, R04-011, and R03-015, received a prophylactic DNA/SeV-Gag vaccine [7]. The DNA used for the vaccination, CMV-SHIVdEN, was constructed from *env*-deleted and *nef*-deleted simian–human immunodeficiency virus $SHIV_{MD14YE}$ [12] molecular clone DNA (SIVGP1) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV chimeric Vpr, and HIV Tat and Rev. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals received a single boost intranasally with 6×10^9 cell infectious units (CIUs) of F-deleted replication-defective SeV-Gag [13,14]. All six *90-088-Ij*-positive animals including three unvaccinated and three vaccinated were challenged intravenously with 1000 50% tissue culture infective doses (TCID50) of SIVmac239 [15] approximately 3 months after the boost. At week 1 after SIV challenge, macaque R03-015 was inoculated with nonspecific immunoglobulin G as previously described [11].

In the second set of experiment, unvaccinated (R06-001) and vaccinated (R05-028) rhesus macaques possessing the MHC-I haplotype *90-120-Ib* were challenged intravenously with 1000 TCID50 of SIVmac239. The latter R05-028 were immunized intranasally with F-deleted SeV-Gag approximately 3 months before the challenge.

In the third, three rhesus macaques received FMSIV plus mCAT1-expressing DNA vaccination three times with intervals of 4 weeks. The FMSIV DNA was constructed by replacing *nef*-deleted $SHIV_{MD14YE}$ with Friend murine leukemia virus (FMLV) *env*, carrying the same SIVmac239-derived antigen-coding regions with SIVGP1, as described before [16]. Vaccination of macaques with FMSIV and a DNA expressing the FMLV receptor (mCAT1) [17] three times with intervals of a week was previously shown to induce mCAT1-dependent confined FMSIV replication resulting in efficient CTL induction while vaccination three times with intervals of 4 weeks in the present study resulted in marginal levels of responses (data not shown). These three DNA-vaccinated animals were challenged intravenously with 1000 TCID50 of SIVmac239 approximately 2 months after the last vaccination.

Some animal experiments were conducted in the Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates, in accordance with the guidelines for animal experiments at the National Institute of Infectious Diseases, and others were in Institute for Virus Research, Kyoto University in accordance with the institutional regulations.

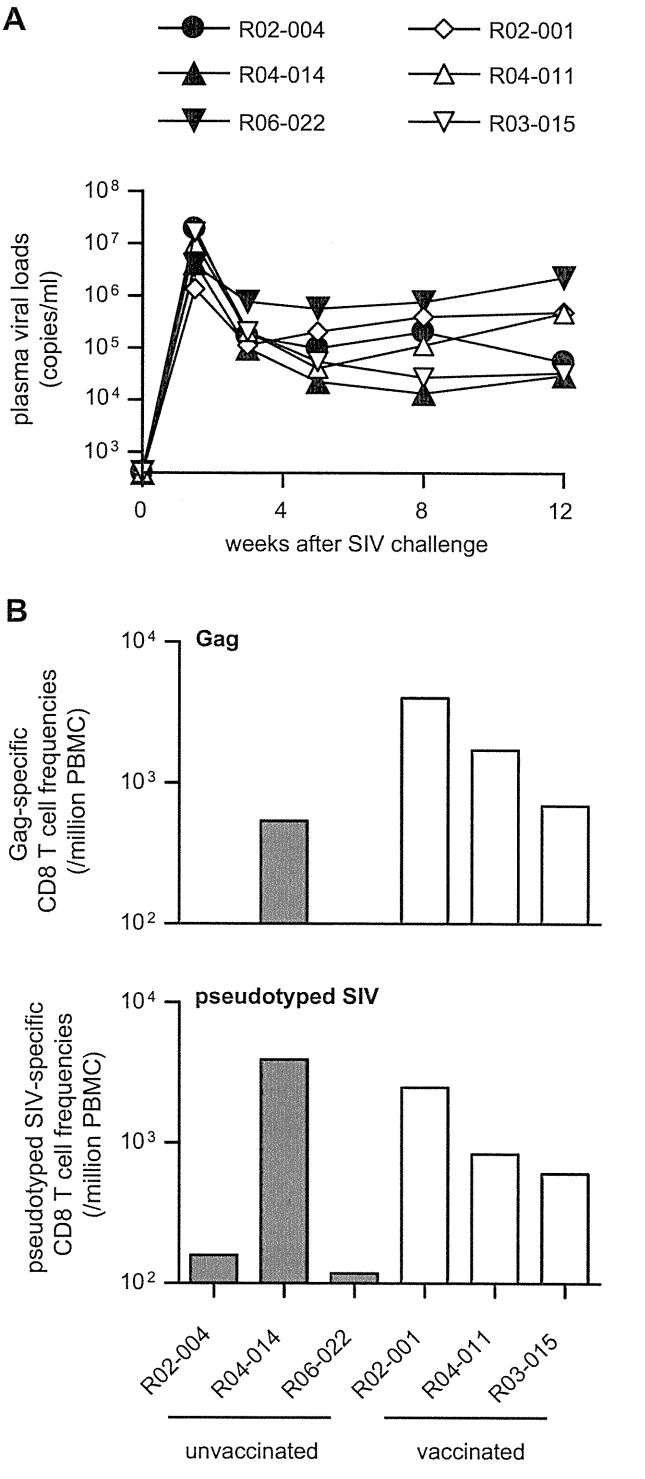

Fig. 1. CTL responses after SIVmac239 challenge in *90-088-Ij*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated (R02-004, R04-014, and R06-022) and DNA/SeV-Gag vaccinated animals (R02-001, R04-011, and R03-015). The viral loads (SIV gag RNA copies/ml) were determined as described previously [7]. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific $CD8^+$ T cell frequencies (lower panel) at week 2 after SIV challenge.

2.2. Analysis of virus-specific CTL responses

We measured virus-specific $CD8^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific

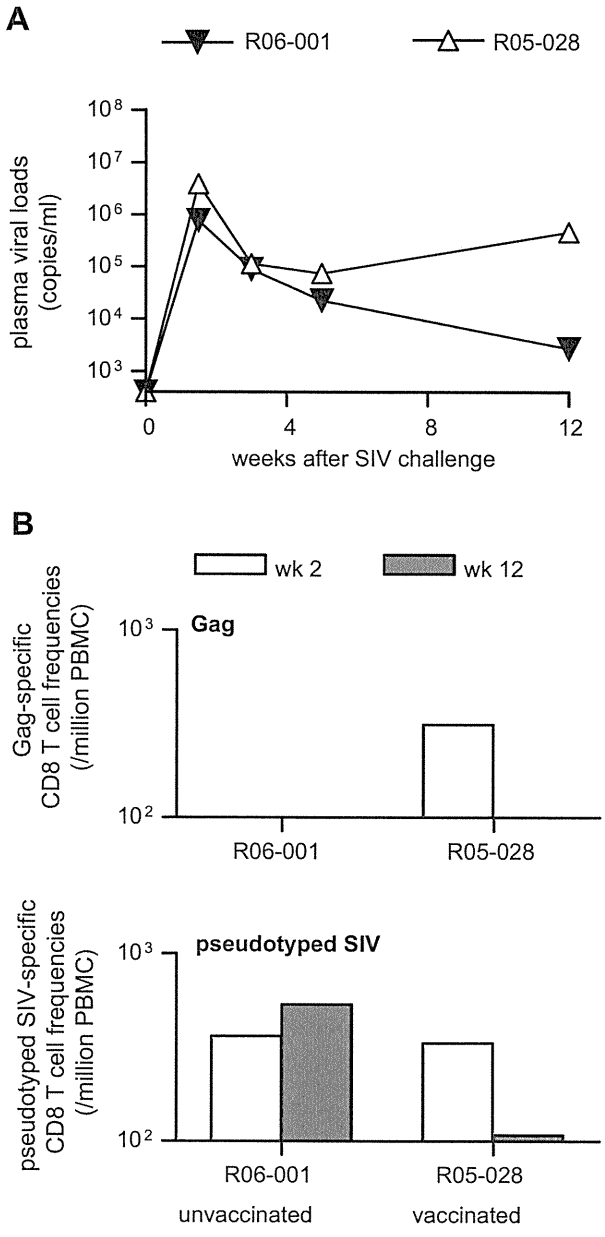

Fig. 2. CTL responses after SIVmac239 challenge in *90–120-Ib*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated R06–001 and SeV-Gag-vaccinated macaque R05-028. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific $CD8^+$ T cell frequencies (lower panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge.

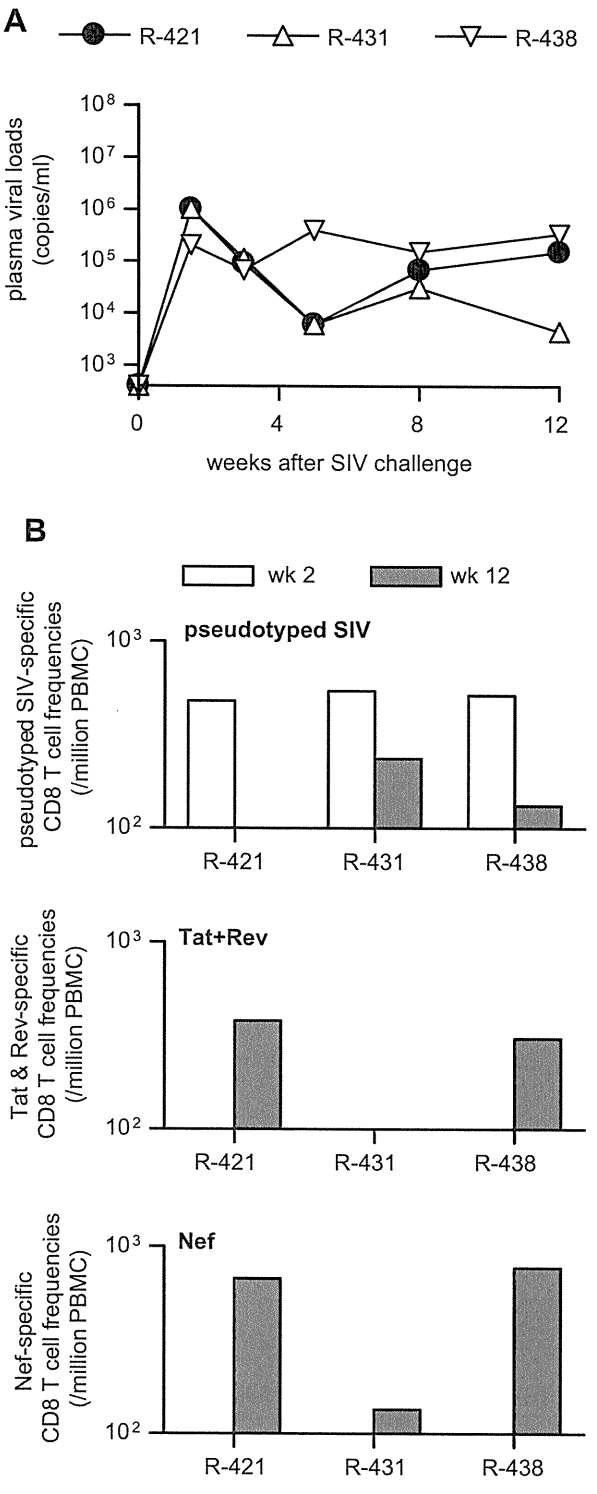

Fig. 3. CTL responses after SIVmac239 challenge in DNA-vaccinated macaques. The DNA used for the vaccination has the SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx and is expected to induce pseudotyped SIV-specific CTL responses. (A) Plasma viral loads after SIV challenge in DNA vaccinated macaques R-421, R-431, and R-438. (B) Vaccine antigen (pseudotyped SIV)-specific (top panel), Tat-plus-Rev-specific (middle panel), and Nef-specific $CD8^+$ T cell frequencies (bottom panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge. In macaque R-438, CTL responses at week 5 instead of week 12 are shown.

stimulation as described previously [18,19]. Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation or a vesicular stomatitis virus G protein (VSV-G)-pseudotyped SIV for pseudotyped SIV-specific stimulation. The pseudotyped SIV was obtained by cotransfection of COS-1 cells with a VSV-G-expression plasmid and SIVGP1 DNA. Alternatively, PBMCs were cocultured with B-LCLs pulsed with peptide pools using panels of overlapping peptides spanning the entire SIVmac239 Tat, Rev, and Nef amino acid sequences. Intracellular IFN-γ staining was performed with a CytofixCytoperm kit (Becton Dickinson, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4, peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ monoclonal antibodies (Becton Dickinson). Specific $CD8^+$ T-cell levels were calculated by subtracting nonspecific IFN-γ^+ $CD8^+$ T-cell frequencies from those after Gag-specific, pseudotyped

	vaccine antigen					non-vaccine antigen										
	Gag				Vif	Vpr	Tat					Rev		Nef		
	165	333	375	376	143	73	23	115	120	122	125	45	50	63	100	124
wk 5																
R- 421					++											
R- 431					+											
R- 438	++		+							++						
wk 12																
R- 421		++			++				+		+	+	+			++
R- 431					+		+			++						
R- 438	++			++		+		++						++	++	

Fig. 4. Viral mutations in DNA-vaccinated macaques. Plasma viral genome sequencing was performed as described previously [18] to determine mutations resulting in amino acid substitutions in SIV Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, and Nef antigens (except for Env) at weeks 5 and 12 in DNA-vaccinated macaques. The amino acid positions showing mutant sequences dominantly (++) or equivalently with wild type (+) are shown. While we found a mutation leading to a lysine-to-arginine alteration at the 40th amino acid in Rev in all animals, this mutation is not shown because the wild-type sequence at this position in the SIVmac239 molecular clone is considered to be a suboptimal nucleotide that frequently reverts to an alternative sequence in vivo [18,23].

SIV-specific, or peptide-specific stimulation. Specific $CD8^+$ T-cell levels lower than 100 per million PBMCs were considered negative.

3. Results and discussion

In our previous SIVmac239 challenge experiments, the prophylactic DNA/SeV-Gag vaccination did not result in viral control in rhesus macaques possessing the MHC-I haplotype *90-088-Ij*. These vaccinated animals showed similar levels of plasma viral loads as those in unvaccinated *90-088-Ij*-positive animals after SIV challenge (Fig. 1A). Analysis of virus-specific $CD8^+$ T-cell responses using PBMCs at week 2 after challenge showed equivalent Gag-specific and pseudotyped SIV-specific (Gag-, Pol-, Vif-, and Vpx-specific) CTL responses in all three vaccinees (Fig. 1B). Pseudotyped SIV-specific CTL responses were also detected in all three unvaccinated animals, but Gag-specific CTL responses were undetectable in two out of the three; even the Gag-specific CTL responses detected in macaque R04-014 were much lower than pseudotyped SIV-specific CTL responses, indicating dominant induction of CTL responses specific for SIV antigens other than Gag (Fig. 1B). Thus, in the acute phase of SIV infection, SIV non-Gag antigen-specific CTL responses were dominantly induced in unvaccinated *90-088-Ij*-positive macaques, whereas vaccine antigen (Gag)-specific CTL responses were dominant in *90-088-Ij*-positive vaccinees.

We then analyzed another vaccinees that failed to control a SIVmac239 challenge; these macaques were vaccinated with SeV-Gag alone or DNA alone. First, we compared post-challenge CTL responses in unvaccinated and SeV-Gag-vaccinated macaques possessing the MHC-I haplotype *90-120-Ib*. Both macaques failed to control SIV replication after challenge (Fig. 2A). In the unvaccinated animal R06-001, Gag-specific CTL responses were undetectable but pseudotyped SIV-specific CTL responses were induced efficiently at weeks 2 and 12 (Fig. 2B). In contrast, Gag-specific CTL responses were induced efficiently at week 2 in the SeV-Gag-vaccinated animal R05-028 (Fig. 2B). At week 12, Gag-specific CTL responses became undetectable while pseudotyped SIV-specific CTL responses were still detectable in this animal. These results indicate that, in the acute phase after SIVmac239 challenge, the unvaccinated *90-120-Ib*-positive macaque dominantly elicited SIV non-Gag antigen-specific CTL responses whereas the SeV-Gag-vaccinated *90-120-Ib*-positive macaque dominantly induced vaccine antigen (Gag)-specific CTL responses.

Next, we analyzed post-challenge CTL responses in three DNA-vaccinated macaques. These animals failed to control SIVmac239 replication after challenge (Fig. 3A). The DNA used for the vaccination and the pseudotyped SIV genome both have the same SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx, thus expected to induce pseudotyped SIV-specific CTL responses. Pseudotyped SIV-specific CTL responses, namely vaccine antigen-specific CTL responses, were induced efficiently at week 2 but diminished after that in all three animals (Fig. 3B). In contrast, Tat/Rev- and Nef-specific CTL responses were undetectable at week 2 but induced later (Fig. 3B). Again, vaccine antigen-specific CTL responses were dominantly induced in the acute phase after SIV challenge and non-vaccine antigen-specific CTL responses were elicited later.

All three animals showed viral genome mutations leading to amino acid substitutions in Gag or Vif at week 5 (Fig. 4). Further analysis indicated that viral mutations in vaccine antigen-coding regions appeared earlier than those in other regions. These results may reflect selective pressure on SIV by vaccine antigen-specific CTL responses dominantly induced in the acute phase, although it remains undetermined whether these mutations are CTL escape ones. Disappearance of vaccine antigen-specific CTL responses at week 12 may be explained by rapid selection of CTL escape mutations in vaccine antigen-coding regions. However, analysis using peptides found Gag-specific CTL responses in macaques R-421 and R-431 that had no gag mutations at week 5 (data not shown), suggesting involvement of immunodominance [20] in the disappearance of vaccine antigen-specific CTL responses at week 12.

In summary, the present study indicates that vaccine antigen-specific CTL responses are induced dominantly in the acute phase after viral exposure, with delayed induction of CTL responses specific for SIV non-vaccine antigens (SIV antigens other than vaccine antigens). While this delay previously-observed in vaccine-based SIV controllers [10] can be explained not only by immunodominance but also by reduction in viral loads, the delay in vaccinated non-controllers in the present study might reflect the immunodominance in CTL responses. Thus, in development of a prophylactic, CTL-inducing AIDS vaccine, it is important to select vaccine antigens leading to effective CTL responses post-viral